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Lipopolysaccharide transport to the cell surface: periplasmic transport and assembly into the outer membrane

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Summary

Gram-negative bacteria possess an outer membrane containing lipopolysaccharide (LPS). Proper assembly of the outer membrane prevents certain antibiotics from entering the cell and also allows others to be pumped out. To assemble this barrier, the seven-protein lipopolysaccharide transport (Lpt) system extracts LPS from the outer leaflet of the inner membrane, transports it across the periplasm, and inserts it selectively into the outer leaflet of the outer membrane. As LPS is important, if not essential, in most Gram-negative bacteria, the LPS biosynthesis and biogenesis pathways are attractive targets in the development of new classes of antibiotics. The previous chapter reviewed the biosynthesis of LPS and its extraction from the inner membrane. This chapter will trace its journey across the periplasm and insertion into the outer membrane.

Introduction

As discussed in the previous chapter [1], the highly impenetrable outer membrane (OM) of Gram-negative bacteria is an asymmetric bilayer consisting of phospholipids in the inner leaflet and lipopolysaccharide (LPS) in the outer leaflet [2]. Each time it divides, a Gram-negative cell must synthesize and transport millions of molecules of LPS, a large, complex glycolipid [3-6]. Within the past few years, a model has emerged to describe how the multi-protein lipopolysaccharide transport system (Lpt) moves its substrate to the cell surface. As shown in Figure 1, following its synthesis inside the cell, LPS is flipped to the outer leaflet of the inner membrane (IM) by ATPase MsbA and then extracted from the IM by LptB₂FGC, an unusual ATP-binding cassette transporter. Following extraction, it traverses a bridge formed by LptA and finally is selectively inserted into the outer leaflet of the OM by the LptDE OM translocon. As there is no ATP in the periplasm, the entire journey following flipping to the outer leaflet of the IM is powered solely by ATP hydrolysis in the cytoplasm [7]. While the previous chapter concerned the extraction of LPS from the IM, this chapter will resume following its release to LptA, reviewing its transport across the periplasm and selective insertion into the outer leaflet of the OM. This chapter will also describe recent efforts to exploit proteins in the LPS biosynthesis and biogenesis pathways as targets for novel antibiotics.

Discovery of the outer membrane complex

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LptD, an ~87 kDa OM beta-barrel, was the first Lpt protein discovered. It was described first as a determinant of membrane permeability, earning the designation *imp*, for increased outer membrane permeability. In a genetic screen in 1989, Benson and collaborators found suppressors containing mutations in *imp* that enabled *Escherichia coli* cells deficient in *lamB* (the beta-barrel required for maltodextrin import) to survive in media containing maltodextrins as the sole carbon source [8]. These *imp* mutants were not only permeabilized to maltodextrins, but also to certain antibiotics and other small molecules that normally cannot cross the OM barrier. Independently, *lptD* was also found to affect organic solvent tolerance (giving rise to its *ostA* designation), again implying a change to membrane structure, although the mechanism at this point was unclear [9].

A more detailed picture of *lptD* emerged following the discovery that it is located immediately upstream of *surA*, a periplasmic chaperone important in the biogenesis of OM proteins [10]. Significantly, the operon containing *lptD* and *surA* was found to be upregulated during the σ^E stress response, which increases the production of OM biogenesis factors in response to envelope stress [11]. Given this suggestion of biogenesis involvement, Braun and Silhavy confirmed the essentiality of *lptD* in *E. coli*, showed that it was co-transcribed with *surA*, and demonstrated that LptD depletion leads to cell chaining followed by lysis [12]. Membrane fractionation experiments showed that LptD depletion generates a novel membrane fraction [12], further proving an essential role for LptD in the biogenesis of the cell envelope.

Tommasen and collaborators performed the key experiments that established the function of LptD in LPS assembly. Exploiting the fact that in *Neisseria meningitidis*, unlike in *E. coli*, LPS is not essential [13, 14], they were able to delete *lptD* and consequently observed dramatically reduced LPS levels [15]. They further confirmed that LptD is essential for LPS assembly specifically in the outer leaflet of the OM.

Using a pull-down with LptD as bait, the Silhavy and Kahne laboratories discovered the other component of the OM translocon, the OM lipoprotein LptE (~20 kDa, formerly known as RlpB) [16]. They found LptE to also be essential in *E. coli*. LptE depletion strains show phenotypes similar to LptD depletions [16].

The identification of the Lpt OM complex and subsequent discovery of the IM complex, described in the previous chapter [1], prompted a host of questions about the mechanism of transport to the OM. Two questions in particular loomed large. First, how does LPS travel across the periplasmic space, devoid of ATP? Second, how does the LptDE complex insert LPS selectively into the outer leaflet of the OM without contaminating the inner leaflet?

Building a bridge between membranes

Clues to the mechanism of how LPS reaches the OM translocon emerged following the discovery of LptA [17], an LPS-binding, periplasmic protein (~19 kDa) whose depletion in *E. coli* results in accumulation of LPS on the periplasmic side of the IM [18-20]. Two competing models arose to describe the role played by LptA [4]. In the first model, inspired by the Lol pathway for lipoprotein transport [21], LptA was proposed to act as a soluble chaperone, shuttling between the IM complex and the OM complex. In the second model, LptA was proposed to form a continuous bridge connecting the IM to the OM.

The first piece of evidence for the bridge model was in place before the discovery of LptA. In a series of experiments with *E. coli* spheroplasts, cells in which the peptidoglycan has been digested and the periplasmic contents released, Tommasen and collaborators observed different requirements for LPS versus lipoprotein transport [22]. Addition of periplasmic extract, which contained soluble chaperone LolA, to the spheroplasts released lipoproteins from the IM, but not LPS. Strikingly, the spheroplasts were capable of MsbA-dependent LPS transport to associated OM fragments in the absence of soluble periplasmic components [22]. Later, evidence of a trans-envelope complex came from Kahne and collaborators, who found that all seven Lpt components co-fractionate and co-purify [23]. The Polissi laboratory also found LptC to form stable complexes with LptA [24].

As shown in Figure 1, crystallographic studies provided a structural model for how the bridge might form. In the presence of LPS, LptA crystallized in long fibrils consisting of two bundles of four LptA proteins stacked N-terminus to C-terminus [25]. A previously uncharacterized fold, the LptA structure consists of 16 consecutive, antiparallel beta-strands that resemble a slightly twisted beta-jellyroll. The structure of the periplasmic domain of LptC (~22 kDa), an LPS-binding, bitopic component of the IM complex, was obtained by the Whitfield laboratory [26]. The periplasmic domain of LptC is very similar to that of LptA and consists of 15 consecutive, antiparallel beta-strands, again resembling a beta-jellyroll.

With the structural data and the knowledge that the periplasmic N-terminal domain of LptD is also classified in the same structural superfamily as LptA and LptC (designated OstA) [2, 27], the Ruiz and Kahne laboratories further probed the interactions between Lpt components. By introducing a photo-activatable amino acid into various positions in LptA, Freinkman *et al.* were able to crosslink LptA to both LptC and LptD in vivo and observed an N- to C-terminal orientation of the domains within the bridge [28]. Bridge formation apparently does not rely on the transmembrane domain of LptC, as cells lacking this domain are still viable [29]. LptA does not interact directly with LptE [30].

These studies, although enabling a consensus about the validity of the bridge model, opened many more questions. For example, it is unknown how many LptA monomers are found in a single bridge. Interestingly, although the structures of LptA and LptC possess many conserved hydrophobic residues in their core, in comparison to mammalian LPS-binding proteins they do not contain an obvious cavity deep enough to fit the lipid A fatty acyl chains of LPS [26]. Tran *et al.* demonstrated in pull-down assays that LptA binds the lipid A core [20], and Okuda *et al.* demonstrated that residues in the grooves of LptA and LptC can in fact crosslink to LPS [7], but it remains an open question exactly how LPS interacts with the bridge. It is also unclear whether it is transported as stream of single molecules or as aggregates. More work is needed to understand exactly how LPS makes its journey across the periplasm.

Assembly of the outer membrane translocon: a plug in a barrel

The assembly of the LptDE OM translocon is a feat that requires the coordination of several pathways. LptD is an OM protein that contains two domains: a C-terminal transmembrane beta-barrel domain and a soluble, periplasmic N-terminal domain [2]. Mature LptD possesses two intramolecular disulfide bonds connecting the C-terminal domain to the N-terminal domain; at least one of these bonds must be present for functionality [31]. LptE is a lipoprotein anchored in the inner leaflet of the OM [32]. Following translation, both pre-LptD and pre-LptE pass unfolded through the Sec translocon [33], but the paths of lipoproteins and beta-barrel proteins diverge at the periplasmic leaflet of the IM. After three acyl chains are attached to the N-terminal cysteine residue of its mature form and its signal peptide is cleaved, LptE is released from the IM by ABC transporter LolCDE, transported across the periplasm by soluble chaperone LolA, and incorporated into the inner leaflet of the OM by lipoprotein LolB [21]. After the signal peptide of LptD is cleaved, it is transported across the periplasm by soluble chaperone SurA and then folded into a beta-barrel in the OM by the BamABCDE complex [34-39].

As it became apparent that both OMP and lipoprotein biogenesis pathways are involved in the assembly of the LptDE translocon, a number of questions developed. How do LptD and LptE interact? When do the essential disulfide bonds in LptD form? How does the cell regulate assembly to ensure production of functional complexes? In particular, LptE emerged to play a critical role in the folding and proper oxidation of LptD.

Initial studies focused on understanding how LptD and LptE interact. Chng *et al.* found that LptD and LptE co-purify in a stable 1:1 complex when a polyhistidine-tag is installed on LptE. In fact, LptD cannot be overexpressed without LptE, and the C-terminal domain of LptD strongly protects LptE from proteolytic degradation [40]. This finding led to the development of the plug-and-barrel model, in which LptE resides in the lumen of the beta-barrel of LptD. Guided by the structures of three LptE orthologs, Freinkman *et al.* introduced a photo-activatable amino acid into LptE at various positions and found many to contact LptD [41]. For one LptE position, they identified the interaction site in LptD to be part of a putative extracellular loop of the barrel, confirming the plug-and-barrel model [41].

The importance of the plug for barrel biogenesis became quickly apparent. Deletion of nine amino acids in LptD around the identified LptDE interaction site compromises translocon biogenesis and leads to OM defects [41]. Seeking to better understand how LptE affects translocon assembly, Chimalakonda *et al.* introduced random mutations into *lptE* and screened for mutants with OM permeability defects [42]. One such mutant carried the *lptE6* allele, which encodes a two-amino-acid deletion that affects LptDE complex formation but not complex stability. Suppressors to *lptE6* that improve LptDE complex formation and restore the barrier function of the OM were found not only in *lptD* but also in *bamA*, which encodes the central component of the Bam complex [42]. This study suggests that LptD needs to interact with LptE to be folded efficiently on the Bam complex.

Assembly of the outer membrane translocon: regulation via disulfide bond formation

The critical role played by LptE in the folding of LptD was elucidated further by detailed studies of disulfide bond formation in LptD. LptD contains four cysteine residues, two in the N-terminal domain (Cys₃₁ and Cys₁₇₃) and two in the C-terminal domain (Cys₇₂₄ and Cys₇₂₅). As described above, mature LptD contains two intramolecular disulfide bonds between the N-terminal domain and the C-terminal domain (Cys₃₁—Cys₇₂₄ and Cys₁₇₃—Cys₇₂₅), and at least one is required for LptD to function [31]. LptD disulfide bond formation is thought to involve the oxidase DsbA, which introduces disulfide bonds into proteins in the periplasm [43, 44]. Deletion of *dsbA*, presumably possible only when other oxidants are present, leads to defects in LptD oxidation [31].

When LptE is limiting, LptD is not oxidized correctly [31], and an LptD species containing a disulfide bond between the first two cysteine residues (Cys₃₁—Cys₁₇₃) accumulates [45]. Mutations in *lptD* also cause accumulation of the Cys₃₁—Cys₁₇₃ product [45]. To understand whether this species is a dead-end product or an intermediate in the assembly of the LptDE complex, Chng *et al.* performed a pulse-chase experiment in which FLAG-tagged LptD was purified from cells pulsed with [³⁵S]-methionine and then chased with cold methionine. As disulfide bond formation affects the migration of LptD on SDS-polyacrylamide gels (SDS-

PAGE) [31], they were able to determine that Cys₃₁-Cys₁₇₃ product is indeed on pathway in wild-type cells; it is the predominant form at the beginning of the chase and converted to mature LptD by the end [45]. Depletion of LptE thus stalls folding of LptD at an intermediate state.

To develop a more detailed picture of LptD folding, the pulse-chase experiment was repeated and six folding intermediates were observed, leading to the model depicted in Figure 2 [45]. LptE is required for disulfide bond rearrangement to form the functional translocon, serving as an important check that both components of the translocon are properly assembled [45]. Furthermore, crosslinking studies have indicated that LptA can connect with LptD only when it possesses at least one of the two mature disulfide bonds, ensuring that bridges are not connected to non-functional translocons [28]. It is interesting that although other plug-barrel systems have been identified, the LptDE system is the only one in which the plug is a separate polypeptide from the barrel [46-48]. Perhaps breaking up the plug and barrel is an elegant solution to ensure that multiple envelope processes are coordinated.

The structure of the OM translocon

Once LPS is transported across the periplasm to the OM translocon, it must be selectively inserted into the outer leaflet of the OM. The mechanism of insertion is a matter of on-going investigation and has been greatly assisted by the recent publications of the structure of the LptDE complex, displayed in Figure 3, by the Huang and Dong laboratories [49, 50]. Consisting of 26 antiparallel beta-strands arranged in a distinctive kidney shape, the C-terminal domain of LptD is the largest beta-barrel crystallized to date. As predicted, the LptD N-terminal domain consists of two sheets of eleven antiparallel beta-strands exhibiting the beta-jellyroll fold found in LptA and LptC [49]. LptE consists of two alpha-helices and four beta-strands [49-51]. Three quarters of LptE is located in the lumen of LptD, and the remainder is positioned on the periplasmic face of the barrel. In addition to directly validating the plug-and-barrel model, the structures also confirmed the LptDE interaction sites predicted by the crosslinking studies discussed above [41].

The structures yield several clues to the mechanism of insertion. Like LptA and LptC, the interior groove of the N-terminal domain of LptD is very hydrophobic, suggesting an interaction with the hydrophobic moiety of LPS [49]. In contrast, the interior of the LptDE barrel is fairly hydrophilic, indicating that the barrel is unlikely to interact directly with this same moiety. The prevailing model is that LPS insertion most likely requires the LptD barrel to open between beta-strands 1 and 26, through which LPS can then roll out [41, 49, 50]. Several conserved proline residues disrupt these strands, leaving only three main-chain hydrogen bonds between them. Molecular dynamics simulations concur that the interface between beta-strands 1 and 26 is the weakest in the barrel [50]. This model is analogous to that proposed by Bishop and van den Berg for beta-barrels PagP and FadL, which are also thought to enable their lipid substrates to diffuse laterally into the membrane via opening of weakly-bonded beta-strands, termed crenels [52-55].

Another important mechanistic question concerns the role played by LptE. Grabowicz *et al.* have demonstrated that the plugging function of LptE is important [56], but it is unclear how its ability to bind LPS is involved in the insertion mechanism. Malojcic *et al.* suggest that its ability to disrupt LPS aggregates may be important if LPS is transported across the periplasm in a continuous stream [51]. Apparently, in *N. meningitidis*, where LPS is not essential, deletion of *lptE* does not affect LPS levels or localization, suggesting that insertion may occur differently in this organism [57]. Additional studies, ideally including structures of the translocon in different conformations, are needed to develop a clearer picture of insertion.

LPS biosynthesis and biogenesis as a drug target

The spread of antibiotic resistance, coupled with a sparse pipeline for new antibiotics, is one of the most serious threats facing medicine today [58, 59]. Antibiotic-resistant Gram-negative bacteria in particular pose a daunting challenge. Four of the six pathogens reported to cause the majority of antibiotic-resistant infections in hospitals (the “ESKAPE” pathogens) are Gram-negative [60], but the most important classes of antibiotics effective against Gram-negative infections were discovered more than 50 years ago. Given the dearth of options, physicians have resorted to using colistin, a drug of last resort due to its toxicity, to treat resistant Gram-negative infections [61].

In this clinical environment, new antibiotics effective against Gram-negative bacteria are desperately needed. The OM is a clear target. In fact, colistin, a polycationic lipopeptide antibiotic, is thought to permeabilize the OM by binding LPS, enabling access to its suspected lethal target, the IM [62]. If a molecule that affects the OM is not lethal on its own, it could be effective in combination with an antibiotic that cannot normally cross the OM barrier. Loss of LPS in organisms in which it is nonessential (*e.g. Acinetobacter baumannii*) renders them less virulent and more susceptible to antibiotics [14, 63-65]. Moreover, molecules that interfere with OM biogenesis may be able to potentiate their own uptake. In the Lpt system, LptB may be an attractive target [66, 67], as it is the only enzyme in the pathway.

Currently, only two proteins involved in the biosynthesis and biogenesis of LPS have been extensively explored as targets for novel therapeutics. The first is LpxC (UDP-3-O-(R-3-hydroxymyristoyl)-GlcNAc deacetylase), a zinc-dependent metalloamidase that catalyzes the committed step in the biosynthesis of the lipid A moiety of LPS [3]. Raetz and colleagues at Merck discovered hydroxamic acid derivatives capable of

inhibiting LpxC by chelating the zinc in its active site [68], and they and others have since developed derivatives with promising antibiotic activity [69]. The other target is LptD. From a library of beta-hairpin peptidomimetics, the Robinson group and colleagues at Polyphor found a compound active in the nanomolar range against *Pseudomonas* spp. that can be crosslinked to LptD in vivo [70]. Although more work is needed to fully establish mechanism of action, a mutation that alters the N-terminal domain of LptD appears to specifically confer resistance to the compound [70], and LptD depletions mirror its effect on LPS localization [71]. The field anxiously waits to see how LpxC and LptD inhibitors perform in clinical trials.

Conclusion

Remarkable progress has been made since the 2004 discovery that LptD is directly involved in LPS transport. Strong evidence has been put forth for the existence of a transmembrane bridge to usher LPS from the IM to the OM. This bridge cannot form unless the OM translocon is properly assembled, which relies on the coordination of the lipoprotein and beta-barrel assembly pathways. The publication of the LptDE translocon structures will enable studies of the mechanism of LPS insertion and further the development of LptD-targeting molecules. Despite this progress, however, much remains unknown. Does the final insertion into the OM require energy? How do LptD and LptE work together to insert LPS? Does the cell regulate LPS transport? If so, how? Finally, can we develop strategies to discover more molecules that interfere with this assembly process?

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Competing Interests

We have no competing interests.

Authors' Contributions

All authors made substantial contributions to conception and design of this review. J.M.M drafted the manuscript and D.J.S, B.W.S., N.R., and D.K. revised it critically for important intellectual content. All authors gave final approval of the version to be submitted.

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Figure Captions

Figure 1. LPS is transported across the periplasm via a transenvelope bridge. (a) Current model for LPS transport. The number of LptA monomers in the bridge is unknown. (b) LptA [25] and the periplasmic domains of LptC [26] and LptD [49] form a filament. Residues highlighted are sites that crosslink between the bridge components [28]. (c) LptA, LptC, and the N-terminal domain of LptD all exhibit a beta-jellyroll fold. Highlighted sites in LptA and LptC crosslink to LPS [7]. (PDB accession numbers: 2R1A, 3MY2, 4Q35.)

Figure 2. Current model for the assembly pathway of the LptDE OM translocon. Several unfolded LptD intermediates exist with varying oxidation states of the four cysteine residues [45]. In the non-functional OM translocon, a disulfide bond exists between C₃₁ and C₁₇₃. In the functional OM translocon, two disulfide bonds are present (Cys₃₁—Cys₇₂₄ and Cys₁₇₃—Cys₇₂₅). Note that LptE is required for disulfide rearrangement.

Figure 3. Structure of the plug-and-barrel complex of LptD and LptE [49]. Disulfide bonds connect the N-terminal domain of LptD to its C-terminal domain. The barrel is thought to open during LPS insertion between beta-strands 1 and 26 (highlighted). (PDB accession number: 4Q35.)